REMARKS

The applicants thank the Examiner for reconsideration of the arguments raised in the office action of 02-09-2006. In the current office action, the Examiner rejected claims 1-6, 8-9, 11-13, 15-17, 19, 27-32, and 34-39 (all pending claims). The Applicants have requested claims 1 and 37 be amended. The applicants also point out that in the response to office action submitted by applications on August 9, 2006, a prior modification of claims 34 and 35 (made on 11-29-2005), namely the replacement of "DNA-PK" with "DNA-PKcs", was not brought forward. The applicants have corrected this error and apologize for any inconvenience this may cause the Examiner. The applicants believe no new matter is introduced as a result of such amendment.

Rejection Under 35 USC 112, First Paragraph

The Examiner rejected claims 1-4 and 37-39 under 35 USC first paragraph for failing to comply with the written description requirement. The applicants have amended claim 1 and 37 in response to this rejection. The applicants respectfully suggest that such amendments render claims 1-4 and 37-39 in compliance with 35 USC 112, first paragraph.

Rejection under 35 USC Second Paragraph

The Examiner rejected claims 4-6, 8-9, 11-12, 15-17, 29-31 and 34-36 under 35 USC second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the Examiner objected to the use of the terms DNA-PKcs and DNA-PK as the sole means of identifying the polypeptides claimed.

With regard to the Examiner's comments, the applicants respectfully point out that the DNA-PK is a well described protein in the literature, the composition and function of which is well known in the art. The DNA-dependent protein kinase (DNA–PK) is a nuclear serine/threonine protein kinase that is activated upon association with DNA. Biochemical and genetic data have revealed DNA–PK to be composed of a large catalytic subunit, termed DNA–PKcs, and a regulatory factor termed Ku. DNA-PKcs was cloned in 1995 by Hartley et al. (Hartley et al., Cell, 82: 849–856, 1995). The Ku factor has also been cloned well before the

priority date of the present application. Therefore, the sequence of DNA-PKcs and Ku were well known in the art as of the filing date.

The Hartley paper referenced above also refers to the polypeptide as DNA-PK. The abstract of the Hartley paper begins: "DNA-dependent protein kinase (DNA-PK), which is involved in DNA double-stranded break repair and V(D)J recombination, comprises a DNA-targeting component called Ku and an approximately 460 kDa catalytic subunit, DNA-PKcs." The applicants have determined that the Hartley paper has been cited in the literature 534, making it, to the applicants' knowledge, the most highly cited paper regarding DNA-PK in the field. As a result of such wide-spread acceptance, the art recognizes the terms DNA-PK and DNA-PKcs.

The applicants have attached a review paper as Appendix A to this response (*The DNA-dependent Protein Kinase*, Smith and Jackson, Genes and Development, 13:916-934, 1999) that details the early work identifying and characterizing the structure and function of DNA-PK. In addition, the applicants point out that the polypeptides referred to in the claim are not known in the art by generic designations, such as p350 (which in fact was an earlier designation for DNA-PKcs). The polypeptides are universally referred to as DNA-PK when referring to the enzyme complex and DNA-PKcs when referring to the catalytically active subunit. Furthermore, applicants were not unable to discover other designations for DNA-PK or DNA-PKcs over the last 10 years. There is no evidence of which the applicants are aware that, as of the filing date of the present application, there were any laboratories or other scientific bodies referring to DNA-PK or DNA-PKcs by other than those names. The Examiner has not presented any evidence that laboratories generally used designations other than DNA-PK or DNA-PKcs when referring to these polypeptides, as of the filing date of the present application. In addition, the specification cites the review article attached as Appendix A and other prior art knowledge that defines what the terms DNA-PK and DNA-PKcs refer to, further removing any ambiguity from the claims. When a fact is well known in the art, the applicant is not required to repeat such material in the patent application and such material is considered to be part of the specification. Therefore, the Applicants respectfully suggest the terms DNA-PK and DNA-PKcs have well defined and

universally recognized meanings in the art and that given the teachings and knowledge of the art and the teachings of the present disclosure, the scope of the claim terms DNA-PK and DNA-PKcs are not indefinite.

The Examiner rejected claims 5-6, 8-9, 11-12, 30-31 and 35-36 under 35 USC second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the Examiner objected to the recitation of the catalytic domain of DNA-PKcs, arguing that the catalytic domain of DNA-PKcs was not defined by the claim and one of ordinary skill in the art would not be reasonably apprised of the scope of the invention.

With regard to the Examiner's comments, the applicants respectfully point out, as discussed above, the DNA-PKcs protein is well known, its activity extensively characterized and the polypeptide has been cloned. After cloning of DNA-PKcs, it was noted that while the amino terminal 3500 amino acids did not share significant homology with known proteins, the carboxy-terminal residues (the final 500 or so amino acids) of DNA–PKcs comprised a catalytic domain that falls into the phosphatidylinositol 3 (PI 3)-kinase family (see Hartley et al. Cell, 82: 849–856, 1995; and Poltoratsky et al. J. Immunol., 155: 4529–4533, 1995). Therefore, given the teaching of the art with regard to the cloning of DNA-PKcs, the nature of the catalytic domain of DNA-PKcs and related family members, one of ordinary skill in the art would understand where the catalytic domain was located and therefore understand a claim limitation that required a product to bind outside of such domain.

As the prior art contained the nucleotide information regarding the location of the catalytic domain of DNA-PKcs, the applicant was not required to put such information in the specification. The federal Circuit held in Capon that "Thus, [w]hen the prior art includes the nucleotide information, precedent does not set a per se rule that the information must be determined afresh." Capon v. Eshhar, 418 F.3d 1349, 1358 (Fed. Cir. 2005). The court went on to explain "The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each

patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the state of knowledge in the field and differences in the predictability of the science." Id. at 1357.

Based on the foregoing, the applicants respectfully suggest that given the teachings and knowledge of the art and the teachings of the present disclosure, the meets and bounds of the claim can be determined and one of ordinary skill in the art would be reasonably apprised of the scope of the claims.

Rejections under 35 USC 101 and 35 USC 112, First Paragraph

The Examiner rejected all pending claims under 35 USC 101 as lacking a specific and substantial asserted utility or a well-established utility. The Examiner also rejected all pending claims under 35 USC 112 first paragraph, since one of ordinary skill in the art would not know how to use the claimed invention. The applicants respectfully disagree with the Examiner's conclusion.

The Examiner states that the application "prophetically" asserts a utility for said DNA repair modulators that specifically bind SEQ ID NO. 16 and single chain antibodies that bind outside of the catalytic domain of DNA-PKcs in methods of inducing cell death or apoptosis in cell culture and in vivo (citing pages 33-34 of the specification in particular). As an initial matter, the applicants respectfully point out the present disclosure provides many more asserted utilities for the DNA repair modulators that specifically bind SEQ ID NO. 16 and single chain antibodies that bind outside of the catalytic domain of DNA-PKcs that are not prophetic. The DNA repair modulators and the single chain antibodies act to inhibit repair of DNA damage, such as, but not limited to, DNA damage induced by ionizing radiation.

For example, the specification clearly recites that the compositions and products of the disclosure have a number of utilities. These asserted utilities, include, but are not limited to: (i) use of the compositions and products disclosed in inhibiting non-homologous end joining and inhibiting DNA repair (page 30, line 20 to page 31, line 20); (ii) use of the compositions and products disclosed in method for inducing cell death or apoptosis in a mammalian cell (page 33,

line 24-29); (iii) use of the compositions and products disclosed in reducing recruitment of DNA DSB repair modulators to the site of DNA DSBs (page 30 line 20 to page 31, line 20); (iv) use of the compositions and products disclosed in developing screening assays and identifying compounds that bind to the sequence of SEQ ID NO. 16 (see page 31, line 22 to page 33, line 23); (v) use of the compositions and products disclosed for the intracellular expression of DNA repair modulators (page 30 line 20 to page 31, line 20); and (vi), use of the compositions and products disclosed in sensitizing cells to ionizing radiation (page 34, lines 16-23). Such asserted utilities relate to the inhibition of repair of DNA damage by the disclosed DNA repair modulators.

These asserted utilities are far from prophetic. The examples also provide actual demonstration of the uses disclosed in the specification. Example 2 demonstrates that the DNA repair modulators disclosed can inhibit DNA end joining and can inhibit DNA-PKcs dependent phosphorylation. Example 4 teaches that the DNA modulators disclosed inhibit DNA repair induced by ionizing radiation (IR) and induce cell death. Example 5 shows that the DNA modulators disclosed activate proteases, such as caspases, that are known to be involved in apoptotic and cell death pathways. Example 6 shows the DNA modulators disclosed inhibit DSB repair. Example 8 shows that the DNA modulators disclosed inhibit repair of DBS in mammalian cells and inhibit recruitment of molecules to the site of the DSB, thereby inhibiting DNA repair and inducing apoptosis or cell death.

The Examiner appears to be discounting the additional utilities asserted by the applicants and examining the application such that the only utility for such DNA repair modulators is in the treatment of a disease, such as cancer. As stated above, the applicants disagree with the Examiner's characterization of the asserted utilities in the present application. However, the applicants assert that the present disclosure does in fact provide a credible specific and substantial utility with regard to the use of the disclosed DNA repair modulators in treating diseases, such as cancer. The specification does teach the use of the compositions containing the DNA repair modulators and products for use in treating a variety of cancers; other uses are also described. The data presented in the examples shows that the compounds inhibit repair of

damaged DNA and thus, based on the evidence presented, one of ordinary skill in the art would expect that the compositions containing the DNA repair modulators would be effective in treating diseases, such as cancer (note the applicants have not asserted a "cure" for diseases such as cancer, simply a treatment of diseases such as cancer). In no part of the present disclosure is there a statement that the uses of the described DNA repair modulators are limited to treating a disease, such as cancer, as suggested by the Examiner.

The applicants also point out that the claims as written do not claim a therapeutic in vivo use of the compositions and products claimed. For example, claim 1 simply states "A composition comprising a DNA repair modulator that specifically binds to the sequence KKYIEIRKEAREAANGDSDGPSYM (SEQ. ID NO.:16), or a portion thereof, and inhibits non-homologous end joining."

The applicants respectfully suggest the claims rejected have a specific and substantial asserted utility. The Utility Guidelines state that an asserted utility is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion. Clearly the asserted utilities stated in the instant application meet this test. The Guidelines further state that an asserted utility is specific if it is specific to the subject matter claimed. In this case, the asserted utilities depend on the nature of the DNA repair modulators disclosed, therefore the utilities are specific. Finally the guidelines state that an asserted utility is substantial if it is practical and based upon a need in the art thereby defining a "real world" use. Examples of such real world use in the Guidelines include a therapeutic method of treating a known or newly discovered disease and an assay method for identifying compounds that themselves have a "substantial utility". Furthermore, Section 2107.01(c) cautions against the confusion that can result when the office attempts to label an invention as a "research tool", an "intermediate" or "for research purposes". This section states that many research tools, such as gas chromatographs, screening assays and nucleotide sequencing techniques have a clear, specific and unquestionable utility. Therefore, the assessment of whether an invention is useful only in a research setting this does not address whether the invention is useful in the patent sense. The MPEP directs the office personnel to

distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm. Section 2107.01(b) speaks to what is meant by a real world context of use. The MPEP states that office personnel must not interpret the phrase "immediate benefit to the public" or similar phrases to require that products or services be immediately available to the public in order to satisfy the utility requirement. Rather any reasonable use identified by the applicants for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to establishing a substantial utility. The asserted utilities stated by the applicants are practical and based upon a need in the art and therefore have a real world use and as such satisfy the substantial requirement with regard to utility.

In the Utility Guidelines referenced by the Examiner (Example 6- Therapeutic Antibodies"), the office examines a hypothetical claim to a therapeutic antibody. The example states "The specification discloses a pharmaceutical composition containing a carrier, a non-antibody protein X and an antibody, said composition being suitable for treating HIV-1 infections." The example further states "The specification as filed does not disclose or provide any evidence that points to an activity for the compositions such that another non-asserted utility would be well established". Exemplary claims 1 and 2 are provided below for discussion:

1. A composition comprising (a) a pharmaceutically acceptable carrier, (b) a non-antibody protein X, and (c) an antibody, said composition being suitable for treating HIV-1 infections.

2. A method of treating an HIV-1 infected subject, which comprises administering to the subject an amount of the composition of claim 1 effective to reduce the rate of spread of HIV-1 infection in the subject.

The example states that there is no well established utility since the record/prior art does not establish an activity for the claimed composition other than the activity asserted in the specification. However, the example, in the analysis of the claims based on the specification, states that in this example, the applicant has made an assertion of utility by disclosing a composition for treating HIV infections and a method for treating a subject infected with HIV.

The example continues to state that the asserted utilities are specific since HIV is a known problem and the disclosed uses depend upon the particular protein disclosed. With regard to substantial utility, the example concludes that the asserted utilities are substantial since the asserted utilities are practical and based upon a need in the art; therefore, the asserted utilities are substantial and "real world". With regard to "real world" utilities, the MPEP 2107.01(b) cautions the office about interpreting the phrase "immediate benefit to the public" to require the invention be currently available to the public in order to satisfy the utility requirement. The MPEP further states that any reasonable use that an applicant has identified for the invention that can be viewed as having a public benefit should be accepted as sufficient, at least with regard to defining a substantial utility. With regard to the credibility of the "specific and substantial utility" of claims 1 and 2, the example concludes that claims 1 and 2 have a specific and substantial utility that is credible.

The claims at issue here are analogous. They claim a composition comprising a DNA repair modulator that has the utilities stated above, such as, but not limited to, binding to SEQ ID NO. 16 (claim 1) or interacting with DNA-PKcs outside the catalytic domain (claim 6) with the stated effects on DNA repair. In the same manner as the above example, the applicants have made an assertion of utility (for example, binding to SEQ ID NO. 16 and inhibiting non-homologous end joining (claim 1), or interacting with DNA-PKcs outside the catalytic domain and inhibiting DNA repair (claim 6)). Each of these asserted utilities is linked to the inhibition of DNA repair, with the inhibition being linked to cell death, apoptosis and other mechanisms. The utility is also specific since the asserted utilities depend on the DNA repair modulators disclosed. Furthermore, the asserted utility is substantial since the asserted utilities are practical based on a need in the art (the need to identify inhibitors of DNA repair. Therefore, the asserted utility is real world. Furthermore, the asserted specific and substantial utility is credible since the claims are directed to subject matter that is credible (as defined in the Guidelines). Therefore, the applicants respectfully suggest the claims rejected each have a well asserted and/or specific and substantial utility that is credible.

The applicants also respectfully suggest the disclosed DNA repair modulators have a well-established utility. The MPEP states that an invention as a well-established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristic of the invention (e.g. properties or applications of a product or process) and (ii) the utility is specific substantial and credible (MPEP 2107).

For example, one of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristic of the invention, such as, but not limited to, the (i) use of the compositions and products disclosed in inhibiting non-homologous end joining and inhibiting DNA repair (page 30, line 20 to page 31, line 20); (ii) use of the compositions and products disclosed in methods for inducing cell death or apoptosis in a mammalian cell (page 33, line 24-29); (iii) use of the compositions and products disclosed in reducing recruitment of DNA DSB repair modulators to the site of DNA DSBs (page 30 line 20 to page 31, line 20); (iv) use of the compositions and products disclosed in developing screening assays and identifying compounds that bind to the sequence of SEQ ID NO. 16 (see page 31, line 22 to page 33, line 23); (v) use of the compositions and products disclosed for the intracellular expression of DNA repair modulators (page 30 line 20 to page 31, line 20); and (vi), use of the compositions and products disclosed in sensitizing cells to ionizing radiation (page 34, lines 16-23). The applicants have clearly shown the beneficial uses of the disclosed DNA repair modulators in the examples.

As only one example, one of ordinary skill in the art would immediately recognize the utility and use of the disclosed DNA repair modulators in the inhibition of non-homologous end joining and the resulting inhibition of DNA repair. The specification describes and the examples show this use.

As discussed above, the applicants also assert that the well-established utility is credible, specific and substantial.

Alternatively, while the applicants continue to assert the application discloses a credible, specific and substantial utility and a well established utility, the applicants point out that an application does not per se require a disclosure of utility in all cases. The MPEP recognizes such

a situation in Section 2107.02(b) where it states "For example, if an applciaiton teaches the cloning and characterization of the nucleotide sequence of a well-known protein such as insulin, and those skilled in the art at the time of filing knew that insulin had a well established utility, it would be improper to reject the claimed invention as lacking utility solely because of the omitted statement of specific and substantial utility." In this case, the application teaches DNA repair modulators that bind to SEQ ID NO. 16 and a region of DNA-PKcs outside of the catalytic domain (similar to the cloning and characterization of insulin in the example above). Likewise, those skilled in the art at the time of filing would be aware that such DNA repair modulators had a well established utility in inhibiting DNA repair just as those skilled in the art at the time of filing knew that insulin has a well established utility.

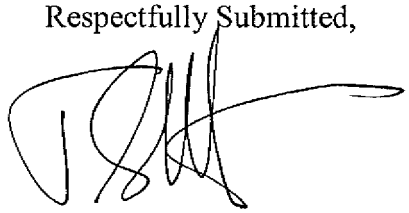
The MPEP in Section 2107.02 states that an applicant need only make one credible assertion of a specific utility for the claimed invention to satisfy 35 USC 101 and 35 USC 112; additional statements of utility, even if not credible, do not render the claimed invention lacking in utility.

Applicants respectfully submit that since the utility rejections are overcome as discussed above, the 35 USC 112 first paragraph rejections are also overcome. In light of the foregoing, applicants respectfully request the Examiner withdraw the rejections.

Conclusion

The above identified applicant respectfully requests the Commissioner of Patents consider the enclosed remarks and enter the following submission into the record, in response to the Examiner's Office Action dated October 17, 2006. Reconsideration in light of this submission is respectfully requested and Applicants respectfully request the application be processed for allowance. If the Examiner requires additional action that may benefit from a telephone call, Applicant invites a call to its attorney of record, T. Gregory Peterson (Reg. No. 45,587) at 205-521-8084. E-mail correspondence and transactions to gpeterson@bradleyarant.com are authorized and encouraged.

Respectfully Submitted,



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Appendix A
Copy of "*The DNA-dependent Protein Kinase*, Smith and Jackson, Genes and Development, 13:916-934, 1999"; 19 pages

REVIEW

The DNA-dependent protein kinase

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The DNA-dependent protein kinase (DNA–PK) is a nuclear serine/threonine protein kinase that is activated upon association with DNA. Biochemical and genetic data have revealed DNA–PK to be composed of a large catalytic subunit, termed DNA–PKcs, and a regulatory factor termed Ku. In recent years, mammalian DNA–PK has been shown to be a crucial component of both the DNA double-strand break (DSB) repair machinery and the *V(D)J* recombination apparatus. In addition, recent work has implicated DNA–PK components in a variety of other processes, including the modulation of chromatin structure and telomere maintenance.

Our DNA is constantly under attack from reactive oxygen intermediates—by-products of the oxidative metabolism we have evolved for energy supply. Reactive oxygen species are capable of producing DNA single-strand breaks and, where two of these are generated in close proximity, DNA double-strand breaks (DSBs). In addition, single- and double-strand breaks can be induced when a DNA replication fork encounters a damaged template, and are generated by exogenous agents such as ionizing radiation (IR) and certain anti-cancer drugs (e.g., bleomycin). DSBs also occur as intermediates in site-specific *V(D)J* recombination, a process that is critical for the generation of a functional vertebrate immune system. If DNA DSBs are left unrepaired or are repaired inaccurately, mutations and/or chromosomal aberrations are induced, which in turn may lead to cell death or, in extreme cases, cancer. To combat the serious threats posed by DNA DSBs, eukaryotic cells have evolved several mechanisms to mediate their repair. In higher eukaryotes, the predominant of these mechanisms is DNA nonhomologous end-joining (NHEJ), also known as illegitimate recombination. DNA–PK plays a key role in this pathway.

Early studies on DNA–PK focused primarily on its biochemistry, and led researchers to speculate on its function as a modulator of transcription. However, this viewpoint took a dramatic change when it was shown that DNA–PK is activated most potently by DNA DSBs, suggesting that it might play a role in recognizing DNA damage. This observation stimulated investigations into the potential role of DNA–PK in DNA repair and led to the identification of cell lines that are radiosensitive due to mutations in DNA–PK components. At around the same time, DNA–PKcs was found to be mutated in cells derived from the radiosensitive and *V(D)J* recombination deficient severe combined immune-deficient (SCID) mouse. The subsequent cloning of the DNA–PKcs cDNA revealed this very large polypeptide to be similar in sequence, over its kinase domain, to an expanding family of proteins involved in controlling cell cycle progression and maintaining genomic stability. Here, we review what is currently known about DNA–PK, with emphasis on recent biochemical analyses that have begun to shed light into how DNA–PK and associated proteins function at the molecular level. We also focus on the results of ablating the function of DNA–PK subunits in both yeast and mice, which have suggested other physiological roles for DNA–PK and its components.

DNA–PK: a historical perspective

A conspicuous property of DNA–PK is that its catalytic activity is triggered upon association with DNA. The ability of double-stranded DNA (dsDNA) to stimulate a protein kinase was first observed in mouse spleen cell nuclear extracts by Ohtsuki et al. (1980), although whether this activity corresponded to DNA–PK is uncertain. Later, a DNA-activated protein kinase was discovered serendipitously by Walker et al. (1985) when studying RNA activation of protein phosphorylation—an RNA sample contaminated with DNA led to the finding that rabbit reticulocyte, human cell, *Xenopus* oocyte, and several murine and amphibian cell extracts are capable of phosphorylating endogenous proteins in a dsDNA-dependent manner. Independently, a DNA-dependent kinase was discovered in HeLa cell extracts as an activity that was capable of phosphorylating SV40 virus large T antigen, transcription factor Sp1, and a variety of other DNA-binding proteins (Carter et al. 1988; Jackson et al. 1990). Intriguingly, these two latter studies noted that the enzyme functions efficiently in the presence of linear but not supercoiled DNA. Through exchange of materials, the three groups concluded that they had identified the same kinase, henceforth termed DNA–PK (Carter et al. 1990; Jackson et al. 1990; Lees-Miller et al. 1990). Initial work indicated that DNA–PK activity copurified with a ~350-kD polypeptide, originally termed 'p350'. Lees-Miller et al. (1990) did note,

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however, that this protein did not always copurify precisely with DNA–PK catalytic activity and found that a high resolution chromatographic step resulted in a p350 preparation dramatically reduced in catalytic activity. This suggested either that p350 did not correspond to DNA–PK or that p350 required an additional polypeptide(s) to function. The latter possibility was shown to be correct through subsequent biochemical studies, which revealed that DNA–PK comprises a large catalytic subunit (p350; now termed DNA–PKcs) and a DNA-targeting component corresponding to the nonspecific DNA end-binding protein Ku (Dvir et al. 1992; Gottlieb and Jackson 1993).

DNA–PKcs is a member of the PI 3-kinase family

Cloning of the DNA–PKcs cDNA revealed that it corresponds to a ~470-kD polypeptide, the amino-terminal ~3500 amino acid residues of which does not appear to have significant homology to other characterized proteins (Hartley et al. 1995). More significantly, however, the carboxy-terminal ~500 residues of DNA–PKcs comprises a catalytic domain that falls into the phosphatidylinositol 3 (PI 3)-kinase family (Hartley et al. 1995; Poltoratsky et al. 1995). Although this suggested initially that DNA–PK might be capable of phosphorylating inositol phospholipids, like certain well-characterized members of the PI 3-kinase family (Toker and Cantley 1997), the available evidence indicates that DNA–PK has protein but not lipid kinase activity (Hartley et al. 1995; Smith et al. 1999). At a similar time to the cloning of the DNA–PKcs cDNA, the genes and cDNAs for a range of other large PI 3-kinase-like (PIKL) proteins were identified and cloned (for reviews, see Zakian 1995; Jackson 1996). These proteins have been shown to be involved in controlling transcription, the cell cycle and/or genome stability in organisms ranging from yeast to man. DNA–PKcs, however, appears to be restricted to higher eukaryotes; clear homologs have been identified in mouse (Araki et al. 1997), horse (Shin et al. 1997), and *Xenopus laevis* (Labhart 1997), but is not present in the genome of *Saccharomyces cerevisiae* and has not been identified in the genomic sequences available for *Caenorhabditis elegans*.

Besides DNA–PKcs, probably the best characterized member of the PIKL family is ATM, the protein deficient in the human neurodegenerative and cancer predisposition condition ataxia-telangiectasia (A-T; for review see Lavin and Shiloh 1997). ATM has been linked intimately to the detection and signaling of DNA damage (for review, see Rotman and Shiloh 1998). ATM homologs also exist in *S. cerevisiae* (Tel1p; Greenwell et al. 1995; Morrow et al. 1995) and *Schizosaccharomyces pombe* (Naito, et al. 1998) and are involved in genome surveillance and in controlling telomeric function. Another PIKL protein involved in genome surveillance is human AT-related (ATR)/FRAP-related (FRP) (Cimprich et al. 1996), together with its homologs in *S. cerevisiae* (Mec1p; Kato and Ogawa 1994; Weinert et al. 1994), *S. pombe* (Rad3; Jimenez et al. 1992; Seaton et al. 1992), and *Drosophila* (*mei-41*; Hari et al. 1995). Human FRAP (Kunz et al. 1993; Brown et al. 1994; Chiu et al. 1994; Sabatini et al. 1994) and its *S. cerevisiae* homologs Tor1p and Tor2p (Heitman et al. 1991; Helliwell et al. 1994), are also in the PIKL family. These proteins control mRNA translation in response to nutrient supply and certain other environmental stimuli, including polypeptide growth factors (Brunn et al. 1997). As with DNA–PKcs, the available data support the proposal that the above PIKL family members are serine/threonine protein kinases but not lipid kinases. A final, apparently more distantly related, member of the PIKL family has been identified recently, and has been named Tra1p in *S. cerevisiae* and TRRAP in humans (McMahon et al. 1998; Saleh et al. 1998). This ~450 kD polypeptide is associated with the SAGA histone acetyltransferase complex that functions in transcriptional control (Apone et al. 1998; Grant et al. 1998; Natarajan et al. 1998). Intriguingly, TRRAP/Tra1p (and its homolog in *C. elegans*) appears to have lost its ability to function as a kinase and most probably acts as a scaffold for proteins involved in chromatin remodeling.

Outside the kinase domain, DNA–PKcs has little or no similarity with other proteins and, besides the presence of a putative leucine zipper motif, which is required for interactions with the high-affinity DNA binding protein C1D (Yavuzer et al. 1998), it has no clear features that might hint at its molecular functions. Nevertheless, the carboxy-terminal quarter of DNA–PKcs has been reported to interact with Ku (Jin et al. 1997). Last year, a significant step forward in understanding the architecture of DNA–PKcs was brought about by cryoelectron microscopy imaging of DNA–PKcs to a resolution of ~20 Å (Chiu et al. 1998). This suggested that DNA–PKcs has an open, pseudo two-fold symmetric structure with a gap separating a crown-shaped top from a rounded bottom. The hollow nature of the DNA–PKcs interior suggests it may interact with DNA via internalization of double-stranded ends through tunnels and cavities identified in the structure. Although the size of the interior of the protein is large, it is thought not big enough to simultaneously internalize the Ku heterodimer and DNA. Therefore, the Ku binding site(s) is most probably located on an exterior surface. Another more recent study has reported the structure of DNA–PKcs at 22 Å resolution by electron crystallography (Leuther et al. 1999). This structure shows DNA–PKcs to be similar to other dsDNA-binding proteins, with it possessing an open channel and an enclosed cavity with three openings large enough to accommodate ssDNA. Biochemical analyses based on this knowledge have suggested that activation of the kinase requires interactions with both double- and single-stranded DNA (Leuther et al. 1999). It will be of great interest to conduct more detailed structural determinations of DNA–PKcs and the DNA–PKcs/Ku complex to learn more about their molecular architectures.

Analyses of the Ku subunits

Ku was first identified as an autoimmune antigen in patients with scleroderma–polymyositis overlap syndrome

(Mimori et al. 1981). The initial and detailed characterization of this protein revealed it to be a highly abundant nonspecific DNA-binding protein comprising two tightly-associated subunits of ~70 and 83 kD (Ku70 and Ku80, respectively; Ku80 is sometimes referred to as Ku86). The cloning of cDNAs and genes for Ku subunits from a variety of species has now taken place and has revealed that both Ku70 and Ku80 exist in organisms ranging from yeast to man (for review, see Dynan and Yoo 1998). Although sequence analyses have so far told us relatively little about the structural organization of the Ku heterodimer, it has recently become apparent that homology exists between Ku70 and Ku80, suggesting that they arose through the duplication and subsequent divergence of a single polypeptide, which presumably functioned as a homodimer (Dynan and Yoo 1998; D. Gell and S.P. Jackson, unpubl.). Each Ku subunit has been reported to possess a leucine-zipper motif, but whether or not these have roles in the overall function or conformation of the proteins is still open to question (Wu and Lieber 1996). Also identified in the sequences of both Ku70 and Ku80 are weak ATP binding site homologies, which may be essential for the proposed ATPase and helicase functions of Ku (Cao et al. 1994; Tuteja et al. 1994; see below). However, the significance of the ATP-binding motifs is unclear, as mutation of these so far have not been found to affect Ku function in vivo (Jin and Weaver 1997; Singleton et al. 1997). Numerous yeast two-hybrid and biochemical studies have been conducted to map the regions of the Ku subunits that make contact with one another and with DNA (Wu and Lieber 1996; Jin and Weaver 1997; Osipovich et al. 1997; Cary et al. 1998; Wang et al. 1998). Although both conflicting and corroborating results have arisen from these studies, it seems apparent that a carboxy-terminal region of ~150 amino acid residues in both Ku70 and Ku80 are essential for dimerization, and that larger regions of both proteins are required for effective interactions with DNA ends. Still to be identified, however, are the precise residues that mediate the extremely strong Ku70–Ku80 interaction, interactions with DNA, and the weaker but critically important interaction with DNA–PKcs.

DNA-binding properties of Ku

The Ku heterodimer was first described as having the apparently unique ability to bind with high selectivity to free dsDNA ends (Mimori and Hardin 1986). The early studies on DNA binding by Ku revealed that it takes place with high affinity and that end binding is independent of the structure or sequence of the end (Mimori and Hardin 1986; Paillard and Strauss 1991; Falzon et al. 1993). Studies have also shown that Ku can bind to a variety of DNA structures; however, it does not bind to closed circular DNA (Dynan and Yoo 1998). Both Ku70 and Ku80 make contact with DNA, although it appears that Ku70 makes the more intimate interactions, with a carboxy-terminal stretch of 73-amino acid residues in Ku70 having been shown to make contact with DNA by Southwestern blot analysis (see Dynan and Woo 1998). Importantly, and consistent with genetic data indicating that Ku70 and Ku80 are functionally dependent on each other (see later section), neither subunit alone can bind DNA effectively (Griffith et al. 1992; Ono et al. 1994; Wu and Lieber 1996; Ochem et al. 1997). Ku has been reported on many occassions to bind DNA in a sequence-specific manner. It now appears that in most cases this was artifactual and resulted from the high abundance of Ku and its unique DNA end-binding properties (see Dynan and Woo 1998 for an illuminating summary of this subject). Nevertheless, at least for the case of Ku binding to the NRE-1 element in the long terminal repeat of the mouse mammary tumor virus, it appears that this example of sequence specific binding by Ku does have some functional basis (Giffin et al. 1996).

In addition to being able to interact specifically with DNA ends, Ku also has the ability to translocate along DNA molecules in an ATP-independent manner (DeVries et al. 1989; Paillard and Strauss 1991). This and related properties have led to models in which the binding of Ku to DNA is likened to the sliding of a bead on a piece of string–DNA ends are not required for binding per se but are required for Ku to bind to or dissociate from the DNA template. Consistent with such a model, Ku can generate footprints at internal sites as well as the termini of linear DNA molecules (Mimori and Hardin 1986; DeVries et al. 1989). In addition, atomic force microscopy studies have revealed the existence of internal as well as DNA end-bound DNA–PK complexes, and have shown that Ku can juxtapose two DNA ends via a DNA looping mechanism (Cary et al. 1997). Finally in this regard, electrophoretic mobility shift assays have been employed to show that Ku can actually transit directly from one linear DNA molecule to another if the termini of the two DNAs are capable of base pairing (Bliss and Lane 1997). That the above DNA end-alignment activities may be of functional significance is suggested by the fact that Ku can stimulate DNA end ligation by eukaryotic DNA ligases in vitro (Ramsden and Gellert 1998).

Other, possibly interrelated functions that have been reported for Ku are helicase and ATPase activities. Tuteja et al. (1994) have shown that Ku preparations possess weakly processive helicase function. Furthermore, it has been reported that the Ku70 subunit contains the ATPase activity and is able to perform helicase function independently of Ku80 (Ochem et al. 1997). Cao et al. (1994) have also reported that Ku possesses weak (as compared to other known enzymes) but significant ATPase function and have shown that this is stimulated by DNA–PK-mediated phosphorylation. Perhaps relevant to the above, Ku70 and Ku80 do have some homology with classical Walker box ATP-binding motifs, although, as noted previously, mutation of these sites has not been found to impair Ku function in vivo.

Mechanism of DNA–PK activation

The original papers establishing Ku as a DNA–PK component revealed that it dramatically stimulates DNA–

PKcs kinase function towards a variety of DNA-bound targets (Dvir et al. 1992, 1993; Gottlieb and Jackson 1993). The DNA–PKcs/Ku complex has been shown to phosphorylate proteins most effectively when it is bound to the same DNA molecule as DNA–PK itself, indicating that part of the activation produced by DNA is through the juxtaposition of DNA–PK and its target (Gottlieb and Jackson 1993). However, DNA also stimulates the ability of DNA–PK to phosphorylate non-DNA-binding peptide substrates, implying that binding to DNA must directly or indirectly induce an activating conformational change in DNA–PKcs. Recent studies with ATP noncompetitive inhibitors of DNA–PK indicate that such a conformational change is unlikely to correspond to an unmasking of the ATP-binding site (Izzard et al. 1999). Protein–DNA cross-linking studies have revealed that DNA–PKcs makes intimate contacts with the DNA, suggesting that DNA might directly induce conformational alterations (Lees-Miller et al. 1990; Gottlieb and Jackson 1993). Consistent with this idea, DNA–PKcs activity can be stimulated to some degree in vitro by DNA in the absence of Ku (Yaneva et al. 1997; Hammarsten and Chu 1998; West et al. 1998). Taken together with data revealing that Ku helps to target DNA–PKcs to DNA (Dvir et al. 1992; Gottlieb and Jackson 1993; Suwa et al. 1994; Chan et al. 1996; Cary et al. 1997; Yaneva et al. 1997; Hammarsten and Chu 1998; West et al. 1998), the available data lead to a model in which Ku recruits DNA–PKcs to DNA, which in turn facilitates DNA–PKcs–DNA interactions that release the catalytic potential of the DNA–PK complex. Such a model is also consistent with physiological data revealing that Ku and DNA–PKcs function, at least in a large part, as an interdependent two-component system (see below). Clearly, many questions regarding the mechanism of DNA–PK activation remain to be answered, including how DNA–PKcs and Ku bind to one another and to DNA, and perhaps most importantly, how only very specific types of DNA structure mediate DNA–PK activation.

Regulation of DNA–PK

As well as DNA–PK being modulated through its interactions with Ku and DNA, it has become apparent that DNA–PK activity and function are likely to also be regulated by a variety of other mechanisms. One area that has received particular attention has been the ability of other proteins to influence the activity of DNA–PKcs and/or the DNA–PKcs/Ku complex. For example, the high-affinity DNA binding protein C1D was identified through the yeast two-hybrid approach as interacting with the putative leucine zipper region of DNA–PKcs (Yavuzer et al. 1998). Notably, DNA-bound C1D can trigger DNA–PK activation in a DNA end-independent manner, possibly via inducing alterations in the structure of the DNA double helix. Although the physiological function for C1D is not yet clear, the observations that it behaves as a component of the nuclear matrix and is induced in response to IR suggests that it could play a role in targeting DNA–PK to specific nuclear regions in response to genotoxic insult. Such a model would be consistent with reports that Ku80-deficient *xrs5* cells are reported to have nuclear envelope and nuclear matrix alterations compared to their wild-type controls (Yasui et al. 1991; Korte and Yasui 1993). Perhaps related to the activation of DNA–PK by C1D, high mobility group (HMG) proteins 1 and 2 have also been shown to be capable of stimulating DNA–PK activation in vitro, hinting to the possibility that DNA–PK activation in vivo is influenced by chromatin context (Watanabe et al. 1994; Yumoto et al. 1998). Another protein–protein interaction implicated in regulating DNA–PK function is that between DNA–PKcs and the Lyn tyrosine kinase, which is capable of disrupting the DNA–PKcs/ Ku complex in vitro (Kumar et al. 1998). Heat shock transcription factor 1 (HSF1) can also stimulate DNA–PK activity in vitro through a mechanism that involves interactions between HSF1 and Ku and weaker interactions between HSF1 and DNA–PKcs (Peterson et al. 1995a; Huang et al. 1997). These findings suggest that HSF1 could cooperate with Ku and DNA–PKcs, possibly through stabilizing interactions between the DNA–PK holoenzyme and DNA. Although the physiological relevance of this interaction is currently unclear, it will certainly be of interest to determine whether DNA–PK-deficient animals or cells display hypersensitivity to heat or altered heat shock responses.

Another example of a protein–protein interaction modulating DNA–PK activity has been revealed through studies on the human Ku80 autoantigen-related protein (KARP-1; Myung et al. 1997, 1998). This protein, which apparently is only present in primates, is expressed from the Ku80 locus and corresponds to a 9-kD amino-terminally extended derivative of Ku80. The KARP-1-specific domain encodes heptad repeats of leucine residues flanked by a basic region, and expression of dominant-negative derivatives of KARP-1 in cells has been reported to result in a derepression of DNA–PK activity and hypersensitivity to IR. These observations, together with the observation that KARP-1 is induced upon irradiation of cells in a p53- and ATM-dependent manner (Myung et al. 1998), suggests that KARP-1 might be involved in latter stages of DNA DSB repair, or may be required to repair lesions that are refractory to the actions of the DNA–PK complex containing the constitutively-expressed form of Ku.

Consistent with its proposed role as a primary DNA damage sensor and not as an inducible downstream effector of DNA damage signaling, DNA–PK is present at relatively high levels (up to 1% of HeLa cell nuclear protein) and its levels do not appear to be regulated strongly by DNA-damaging agents (Lee et al. 1997). However, rodents have much lower levels of DNA–PKcs, Ku, and DNA–PK activity than primate cells (Anderson and Lees-Miller 1992; Finnie et al. 1995). Indeed, it is notable that DNA–PK levels in a variety of species correlate well with the species' life-span, suggesting that elevated DNA–PK levels in longer-lived organisms is a mechanism to enhance genomic stability. Despite DNA–PK being constitutively present, its activity does appear to

be modulated throughout the cell cycle (Lee et al. 1997). Furthermore, elevated levels or activity of Ku have been reported in response to cellular growth state (Cai et al. 1994) possibly reflecting the increased need for efficient DNA DSB repair in rapidly dividing cells. DNA–PK activity is found to be induced to some extent when lymphoid cells are induced to undergo site-specific *V(D)J* recombination or switch recombination (Grawunder et al. 1996); two processes that rely on DNA–PK function (e.g., see Rolink et al. 1996; Casellas et al. 1998; Manis et al. 1998). However, as this result was obtained from cells in culture, it remains unclear if this activation of DNA–PK occurs in the whole animal. In addition, decreased vitamin D receptor expression has been found to reduce DNA–PKcs mRNA levels, although the reason for this is currently obscure (Dabrowski et al. 1998).

The recent cloning and analysis of the 5′-untranslated region of the DNA–PKcs gene has revealed it to have no TATA or CCAAT box sequences and to contain potential binding sites for the ubiquitous transcription factor Sp1—features associated with 'housekeeping genes' (Connelly et al. 1998). It will be of interest to study the potential transcriptional induction of DNA–PK components in response to the aforementioned stimuli. Another potential mode for DNA–PK regulation, suggested from the cloning of its cDNA, is at the level of pre-mRNA splicing. The initial DNA–PKcs cDNA sequence lacked an exon of 93 base pairs that was found in subsequent studies (Hartley et al. 1995; Poltoratsky et al. 1995; Connelly et al. 1996). Although it is possible that this reflects aberrant rather than differential splicing in the initial clone, the fact that the differentially spliced exon encodes a part of the catalytic domain suggests that its differential usage could have an important regulatory function.

DNA–PK activity also appears to be regulated by post-translational modification. For example, autophosphorylation of DNA–PKcs in vitro has been shown to induce its dissociation from Ku and result in inhibition of DNA–PK catalytic function (Chan and Lees-Miller 1996). Another DNA–PKcs-phosphorylation event that has been reported to dissociate the DNA–PKcs–Ku complex is that mediated by the c-Abl proto-oncogene product (Jin et al. 1997; Kharbanda et al. 1997). Because c-Abl is itself induced in response to IR in a manner that is reported to be DNA–PK dependent (Kharbanda et al. 1997), this suggests the existence of an autoregulatory negative feed-back loop that might lead to repression of DNA–PK activity after the appropriate DNA damage signaling and/or repair pathways have been initiated. Another post-translational modification that has been found recently to affect DNA–PK activity in vitro is ADP–ribosylation mediated by the DNA repair-associated enzyme poly-ADP(ribosyl) transferase (PARP), which can stimulate the ability of DNA–PK to phosphorylate some protein substrates (Ruscetti et al. 1998). Although the physiological relevance of this is currently unclear, the fact that PARP activity is also induced by DNA damage suggests that it may be a way of coordinating the activities of the DNA–PK and PARP systems.

Perhaps the most striking characterized examples of regulating DNA–PK activity are its inactivation during programmed cell death and upon viral infection. During apoptosis, DNA–PKcs is specifically cleaved by caspase-3 or a caspase-3-like protease with subsequent loss of its kinase potential (Casciolarosen et al. 1995; Han et al. 1996; LeRomancer et al. 1996; Song et al. 1996). This DNA–PK inactivation is probably to prevent signaling from or repair of the degraded genomic DNA that is produced during the latter steps of the apoptotic pathway. Furthermore, as apoptosis is an energy-dependent process, DNA–PK inactivation might prevent the massive activation of this highly abundant kinase by fragmented genomic DNA and the subsequent depletion of ATP reserves. Interestingly, although neither Ku subunit is a target for the apoptotic proteases, a loss of Ku protein has been observed in apoptotic lymphocytes and in myeloid cells destined to undergo apoptosis (Ajmani et al. 1995). A negative regulation of DNA–PK function by protein kinase C δ in apoptotic cells has also been proposed (Bharti et al. 1998). In a different scenario, DNA–PKcs has been found to be degraded, apparently via a proteosomal mechanism, during herpes simplex virus (HSV) type 1 infection of mammalian cells (Lees-Miller et al. 1996; Parkinson et al. 1999). Although other possibilities exist, this suggests that inhibiting DNA–PK function aids virus replication and/or packaging. In line with this idea, at low titers, HSV has been reported to replicate more efficiently in a DNA–PKcs null cell line compared to the DNA–PK positive cells (Parkinson et al. 1999).

Manipulation of DNA–PK activity by exogenous agents

Ku has the ability to bind to artificially derived RNA molecules with an affinity as great as that observed for dsDNA (Yoo and Dynan 1998). Binding to these RNAs does not result in DNA–PK activation, meaning that they have the potential to be developed into agents that can regulate DNA–PK function in vitro or in vivo. The sequence similarity between DNA–PKcs and PI 3-kinases has also provided a route to identify DNA–PK inhibitors. Thus, Wortmannin, a classical PI 3-kinase inhibitor, was found to inhibit DNA–PK with an IC50 of ~250 nM, around two orders of magnitude higher than is required for PI 3-kinase inhibition (Hartley et al. 1995). A more detailed analysis has shown that, similar to PI 3-kinases, DNA–PK is inhibited by Wortmannin in a non-competitive manner, and that Wortmannin is able to bind covalently to the kinase active site (Izzard et al. 1999). Wortmannin is capable of binding to DNA–PKcs in vivo (Izzard et al. 1999) and has been shown to radiosensitize mammalian cells (Price and Youmell 1996; Boulton et al. 1996; Rosenzweig et al. 1997; Hosoi et al. 1998). Other PI 3-kinase inhibitors, such as LY294002, quercitin, quercitrin, and rutin, have also been shown to inhibit DNA–PK activity, with LY294002 being demonstrated to radiosensitize cells (Rosenzweig et al. 1997; Izzard et al. 1999). Another compound that has potential as a DNA–PK inhibitor is OK1035 (Take et al. 1995, 1996). Although somewhat nonspecific in nature, these

compounds represent useful tools for elucidating the functions of DNA–PK in DNA repair and other processes, both in vivo (see above references) and in vitro (Gu et al. 1996, 1998; Baumann and West 1998). In addition, they might serve as starting points for the identification of more specific inhibitors of DNA–PK and/or the other related PI 3-kinases for academic research or for the development of pharmacologically active therapeutic agents.

Role of DNA–PK and associated factors in DNA NHEJ

The involvement of DNA–PK in DSB repair became evident from analyses involving a specific series of mutant rodent cell lines. Early studies into these cells found them to be hypersensitive to IR and radiomimetic agents with little or no cross sensitivity to other types of DNA damaging agent, and showed them to be defective in the repair of chromosomal DNA DSBs (for review, see Zdzienicka 1995). Subsequent cell fusion studies allowed these cells to be placed into three distinct complementation groups, termed IR4, IR5, and IR7, and the human genes complementing them were preassigned to the *XRCC* nomenclature (X-ray cross-complementing; *XRCC4* is the gene that complements cells of IR4, *XRCC5* complements IR5, and *XRCC7* complements IR7; Thompson and Jeggo 1995; Zdzienicka 1995).

In late 1994 and early 1995, a series of reports were published showing that cells of IR5 lack Ku DNA end-binding activity and can be complemented by the gene for Ku80 (Getts and Stamato 1994; Rathmell and Chu 1994; Smider et al. 1994; Taccioli et al. 1994; Boubnov et al. 1995; Finnie et al. 1995). Prompted by the above findings, several groups then established that cells of IR7 also lack DNA–PK activity; in this case the defect being complemented by the gene for DNA–PKcs (Blunt et al. 1995; Kirchgessner et al. 1995; Peterson et al. 1995b). Consistent with this, a radiosensitive human cell line (MO59J) was found to be defective in DNA–PKcs expression and DNA–PK activity (Lees-Miller et al. 1995). Subsequent work showed cells of IR5 and IR7 to harbor inactivating mutations in the genes for Ku80 and DNA–PKcs, respectively, and revealed that inactivation of Ku80 leads to a dramatic destabilization of both itself and Ku70 (Errami et al. 1996, 1998a,b; Blunt et al. 1996; Danska et al. 1996; Araki et al. 1997; Singleton et al. 1997; Peterson et al. 1997; Fukumura et al. 1998; Priestley et al. 1998). It was therefore concluded that mutations in Ku80 or DNA–PKcs lead to IR hypersensitivity, that *XRCC5* and *XRCC7* encode Ku80 and DNA–PKcs, respectively, and that DNA–PK is a crucial component of the mammalian DNA DSB repair apparatus. None of the original rodent cell lines was defective in Ku70, but it was assumed that cells lacking it would have a similar phenotype to those in IR4–7. This has been confirmed by targeted disruption of the gene for Ku70 in mouse cells, allowing such cells to be designated IR6 and the gene for Ku70 to be designated *XRCC6* (Gu et al. 1997). In other work, the *XRCC4* gene product was cloned, and IR4 cells were shown to be deleted for this gene (Li et al. 1995). Although the sequence of the 334-amino acid XRCC4 protein did not initially yield insights into its mode of action, subsequent studies revealed that it forms a tight and specific association with DNA ligase IV (Critchlow et al. 1997; Grawunder et al. 1997), a protein which has itself been revealed recently to function in DNA NHEJ (Barnes et al. 1998; Frank et al. 1998; Grawunder et al. 1998).

Under most circumstances, the predominant mechanism for DNA DSB repair in mammalian cells is that of NHEJ, and it is in this process that the IR4–7 mutants are defective. Characteristically, NHEJ does not need extensive homologies between the recombining DNA molecules nor does it require an undamaged DNA partner; it is therefore distinct from the well-characterized homologous recombination pathway of DSB repair (for reviews, see Chu 1997; Critchlow and Jackson 1998; Kanaar et al. 1998). In contrast to the situation in mammalian cells, *S. cerevisiae* mainly repairs DSBs by homologous recombination—this requires genes in the *RAD52* epistasis group (Kanaar et al. 1998). Nevertheless, recent work has shown yeast to have a NHEJ pathway that is highly related to that in higher eukaryotes. Thus, yeast possesses homologs of Ku70 and Ku80 (Yku70p and Yku80p, respectively; also termed Hdf1p and Hdf2p, respectively) that exist in a heterodimeric complex. Furthermore, loss of either yeast Ku subunit leads to IR hypersenstivity in *rad52* mutant backgrounds and causes NHEJ defects as ascertained by an in vivo plasmid repair assay (Boulton and Jackson 1996a,b; Feldmann et al. 1996; Mages et al. 1996; Milne et al. 1996; Siede et al. 1996). Yeast also contains homologs of XRCC4 and DNA ligase IV (Lif1p and Lig4p or Dnl4p, respectively), and these have been shown to function in the Ku pathway of NHEJ (Schar et al. 1997; Teo and Jackson 1997; Wilson et al. 1997; Herrmann et al. 1998; Ramos et al. 1998). No clear homolog of DNA–PKcs exists in the *S. cerevisiae* genome however, suggesting either that its functions are not conserved or are mediated by other proteins.

Recently, several other yeast NHEJ components have been identified, including the nuclease complex containing Rad50p, Mre11p, and Xrs2p (Milne et al. 1996; Boulton and Jackson 1998). Human homologs of Rad50p and Mre11p have also been identified and have been linked to DNA DSB repair by way of the fact that they become targeted to sites of IR-induced damage in vivo (Maser et al. 1997; Nelms et al. 1998). Moreover, it has been established that human Rad50 and Mre11 exist in a complex with NBS1 (also called Nibrin), a protein whose deficiency leads to the rare human genetic disorder called Nijmegen breakage syndrome (NBS) (Carney et al. 1998; Matsuura et al. 1998b; Varon et al. 1998). NBS is characterized by chromosomal instability, developmental abnormalities, and cancer predisposition (for review, see Featherstone and Jackson 1998). In addition, defects in NBS1 are reported to lead to impaired induction of p53 in response to IR, thus providing an exciting potential linkage between DNA DSB rejoining and DNA damage signaling (Jongmans et al. 1997; Matsuura et al. 1998b). Three other yeast proteins shown to be required for effi-

cient NHEJ are the heterochromatin components Sir2p, Sir3p, and Sir4p (Tsukamoto et al. 1997; Boulton and Jackson 1998). An interaction between yKu70p and Sir4p in the yeast two-hybrid system (Tsukamoto et al. 1997), has suggested that Ku recruits the Sir protein complex to DNA DSBs in vivo and that this might contribute to NHEJ, either by preventing access to nucleases or by facilitating the juxtaposition of the two DNA termini via chromatin condensation. Although such models are attractive, recent work from Rine and colleagues has cast doubt on a direct involvement of the Sir proteins in NHEJ and has suggested that their effects are, at least in part, mediated by influencing the mating type status of the yeast cell (Åström et al. 1999).

There are a variety of other ways in which Ku and DNA–PKcs might function in DNA NHEJ (for more extensive discussions, see Chu 1997; Critchlow and Jackson 1998; Kanaar et al. 1998). Most obviously perhaps, the fact that Ku can bind with great avidity to DNA ends suggests that it directly recognizes DNA DSBs in vivo and, in higher eukaryotes, recruits DNA–PKcs to such sites. Once bound, Ku or the DNA–PK complex might then protect the ends from nucleolytic degradation, as suggested by the ability of Ku-containing complexes to exclude nucleases in vitro (Mimori and Hardin 1986; DeVries et al. 1989; Gottlieb and Jackson 1993). Consistent with this idea, linear DNA transfected into IR5 cells is more susceptible to end degradation than in control cells (Liang and Jasin 1996), and the rare NHEJ products that are generated in mammalian or yeast cells lacking Ku have generally suffered large deletions of terminal sequences before ligation has taken place (for example, see Taccioli et al. 1993; Boulton and Jackson 1996b). Nevertheless, and as discussed later, *V(D)J* recombination intermediates are relatively stable in the absence of Ku or DNA–PKcs, indicating that in this case at least, Ku or DNA–PKcs are not required for DNA end stabilization (Zhu et al. 1996). Another way that Ku/DNA–PKcs could potentiate end ligation is by tethering two DNA ends together. Indeed, Ku is able to promote interactions between two DNA termini (Cary et al. 1997) and can enhance end ligation by eukaryotic DNA ligases in vitro (Ramsden and Gellert 1998). Notably, in situations in which NHEJ cannot occur simply by the direct ligation of mutually complementary 5′- or 3′-overhanging termini, repair products tend to suffer short deletions and become rejoined at sites of short direct repeats of microhomology. It is tempting to speculate that the weak helicase functions of Ku could play a role in dissociating the two strands of the DNA ends to allow such microhomology alignments to be produced, although it should again be noted that no clear phenotype has yet been described for Ku proteins mutated in the proposed helicase motifs (Jin and Weaver 1997; Singleton et al. 1997). Furthermore, it is clear that microhomology directed repair does occur in Ku80 knockout cells (Boguе et al. 1997). Therefore, if Ku does play a role in this pathway, it cannot be essential.

Once positioned at the DNA DSB, Ku and DNA–PKcs might then recruit other NHEJ factors, or, in the case of the DNA–PK holoenzyme, might regulate the activities of these components by phosphorylation. Suggestive that this may indeed be the case are the observations that DNA–PK is able to phosphorylate XRCC4 in vitro and that the XRCC4/ligase IV complex can interact directly or indirectly with DNA–PK in crude nuclear extracts (Critchlow et al. 1997; Leber et al. 1998). Another complex that might be recruited and/or activated by Ku or DNA–PKcs is that containing Rad50, Mre11, and NBS1 (Rad50p, Mre11p, and Xrs2p in yeast), whose nuclease activities may be critical in 'tidying up' damaged DNA termini before they can be ligated together (Furuse et al. 1998; Paull and Gellert 1998; Trujillo et al. 1998). Recent data have indicated that the endonuclease function of Mre11p is not needed for NHEJ of restriction enzyme-generated DNA DSBs (Moreau et al. 1999), but whether this is also the case for the repair of noncomplementary ends or IR-induced damage has not been reported. Finally in regard to the mechanism of Ku and DNA–PKcs action in NHEJ, it is possible that they act to dissociate repair factors from the DNA after their job is complete, or help to remove from the DNA other proteins, such as recombination factors that might block the repair process (Zhu et al. 1996). In this context, it is noteworthy that DNA–PK is capable of repressing transcription by RNA polymerase I in vitro (Kuhn et al. 1995; Labhart 1995). Also, autophosphorylation leads to the dissociation of DNA–PKcs from DNA-bound Ku (Chan and Lees-Miller 1996). If such an autophosphorylation were to take place in *trans*, it could provide a mechanism for removing DNA–PKcs only when two DNA ends are brought into close proximity. In addition to possibly triggering repair-associated functions of Ku, this could provide an elegant mechanism for coupling repair to inactivation of DNA–PK-mediated DNA damage signaling (discussed below).

To help us in understanding the molecular details of DNA NHEJ, the establishment of biochemical systems that accurately reflect this process will be of importance. A potentially major step towards this goal is the recent development of a mammalian cell-based system that appears to be dependent on DNA–PKcs, Ku, and the ligase IV/XRCC4 complex and also requires other, as-yet-uncharacterized, components (Baumann and West 1998). A recently developed *Xenopus* NHEJ system also appears to be dependent on functional DNA–PK catalytic activity (Gu et al. 1996; 1998). In addition to allowing the identification and characterization of NHEJ components, such systems may be of great use in studying the control of NHEJ during the cell cycle and in analyzing interfaces between NHEJ and other processes, such as transcription and the control of chromatin structure.

Role of DNA–PKcs and Ku in DNA-damage signaling

Despite DNA–PK having been identified for >10 years, the nature of its physiological target(s) is still uncertain. Numerous in vitro substrates have been identified, which include a variety of transcription factors, most notably p53 (discussed in more detail below), the RNA

polymerase II large subunit carboxy-terminal domain (CTD) and chromatin components (for review see Anderson and Lees-Miller 1992). However, whether any or all of these are functional DNA–PK targets in vivo is not yet clear. The 34-kD subunit of replication protein A (RPA) is also a good candidate for a DNA–PK target (Brush et al. 1994) although conflicting reports exist in the literature as to whether it is indeed phosphorylated by DNA–PK in vivo (Boubnov and Weaver 1995; Fried et al. 1996). Other potential DNA–PK targets include components of the DNA NHEJ machinery, such as XRCC4, which is an effective substrate for DNA–PK in vitro and is known to be a phosphoprotein in vivo (Critchlow et al. 1997; Leber et al. 1998). Furthermore, as discussed above, both Ku subunits and DNA–PKcs are subject to DNA–PK phosphorylation in vitro.

Key to elucidating the functional consequences of DNA–PK-mediated phosphorylation events will be the identification and subsequent mutagenesis of phosphorylation sites. Such studies will be facilitated by DNA–PK target consensus sequences having been defined. The available data indicate that DNA–PK has a marked preference for Ser or Thr residues preceding (and to a lesser extent preceded by) a Gln residue, and that phosphorylation is potentiated by adjacent acidic residues and tends to be inhibited by basic residues (Anderson and Lees-Miller 1992; Bannister et al. 1993). Unfortunately from a predictive standpoint, DNA–PK does not recognize all sequences conforming to this consensus, probably due to conformational constraints. It is also clear that not all DNA–PK target sites conform to this consensus.

Being a DNA damage-activated protein kinase, DNA–PK is inherently well suited to functioning in DNA damage signaling. Thus, DNA–PK activation in response to IR or other agents could trigger signaling pathways that result in apoptosis or cell cycle checkpoint arrest—such responses being designed to prevent the proliferation of potentially mutated cells or to allow DNA repair to occur prior to DNA replication or mitosis (for review, see Elledge 1996). Consistent with this idea, an efficient target for DNA–PK in vitro is p53, a factor whose levels and activity are induced in response to DNA damage and which plays key roles in triggering apoptosis or cell cycle arrest in such circumstances (for review, see Ko and Prives 1996). Furthermore, DNA–PK phosphorylates serine 15 and serine 37 of p53 in vitro (Lees-Miller et al. 1990, 1992), and this has been reported to destabilize interactions between p53 and Mdm2, a protein that negatively regulates p53 in vivo by targeting it for ubiquitin-mediated proteolysis (Haupt et al. 1997; Kubbutat et al. 1997; Shieh et al 1997). Initial analyses of cells defective in DNA–PK components have, however, shown them to have intact DNA damage checkpoints and to be capable of mediating p53 stabilization in response to IR (Bogue et al. 1996; Fried et al. 1996; Guidos et al. 1996; Huang et al. 1996; Nacht et al. 1996; Candeias et al. 1997; Rathmell et al. 1997; Shieh et al. 1997). Nevertheless, recent work using certain DNA–PKcs defective cell lines has indicated that DNA–PK activation in response to DNA damage is necessary, but not sufficient, for the activation of sequence specific DNA binding by p53 by an as yet undefined mechanism (Woo et al. 1998). However, new data now exist questioning the interpretation of this study (M. Hubank and G.Wahl pers. comm.). The above studies indicate that DNA–PK either does not regulate p53 through serine 15 phosphorylation or does so in a redundant manner with other proteins. In this regard, it has been shown recently that the DNA–PK related proteins ATM and ATR are also capable of phosphorylating p53 on serine 15 in vitro (Banin et al. 1998; Canman et al. 1998; Khanna et al. 1998), and serine p53 phosphorylation in response to DNA-damaging agents is debilitated in cells impaired or lacking ATM or ATR function (Kastan et al. 1992; Tibbets et al. 1999). Thus, it may be that ATM, ATR, and possibly DNA–PK signal different but partially overlapping types of DNA damage to a common p53 effector pathway (Fig. 1). Notably, DNA–PK has been demonstrated to be capable of phosphorylating Mdm2 in vitro (Mayo et al. 1997), sug-

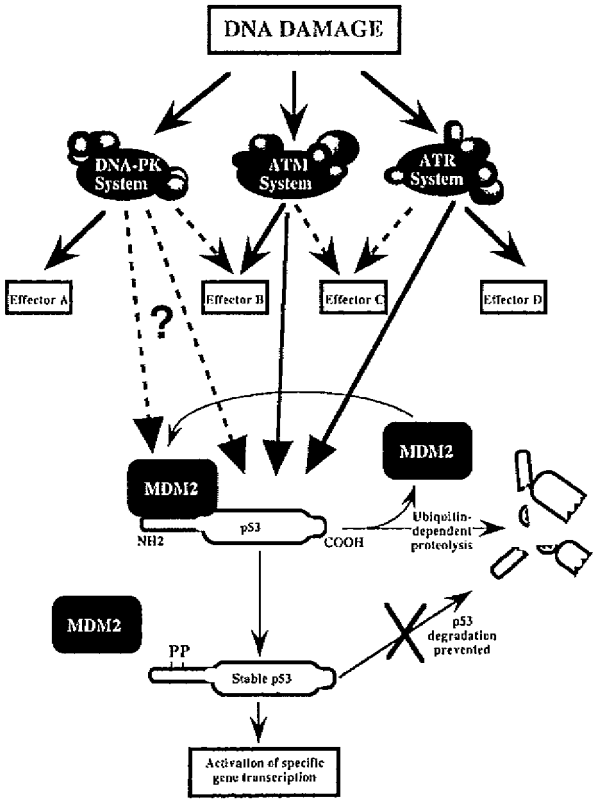

Figure 1. A hypothetical model whereby signaling to p53 by the DNA–PK, ATM and ATR systems leads to phosphorylation at the amino terminus of p53. These phosphorylation events result in the disruption of the p53/MDM2 interaction, and hence lead to p53 protein stabilization and to p53-dependent downstream events. The three DNA damage responsive systems may also signal to other effectors. The arrow from DNA–PK to Mdm2 indicates another potential mechanism by which DNA–PK and its relatives might modulate p53 activity or stability.

gesting that this might be an alternative mechanism by which DNA-PK, ATM, or ATR regulate p53 activity.

Knockout mouse models for DNA-PK components

The first mutant mouse model for analysing DNA-PK function was the spontaneously arising SCID (severe combined immunodeficiency) mouse (Bosma and Carroll 1991). The SCID mouse has a nonfunctional immune system, due to an almost complete lack of mature B and T lymphocytes. Analysis of the SCID defect revealed that it occurs at the level of site-specific *V(D)J* recombination (Lieber et al. 1988). Briefly, this process involves the rearrangement, by a cut-and-paste mechanism, of variable (*V*), diversity (*D*), and joining (*J*) segments of immunoglobulin and T-cell receptor genes, leading to the production of nonfunctional signal joints and to coding joints that encode the highly variable-antigen binding regions of the antigen receptors. The first enzymatic step of *V(D)J* recombination is the formation, by the RAG1 and RAG2 proteins (McBlane et al. 1995), of DNA DSBs between a pair of signal and coding segments. This reaction yields blunt-ended signal termini and coding termini that have covalently sealed hairpin structures (for review, see Ramsden et al. 1997). The second stage of *V(D)J* recombination, in which the RAG proteins appear to play a role (Agrawal and Schatz 1997), involves ligation of the recombination intermediates. For the signal ends, this appears to take place through simple end joining, whereas coding joining is more complex and requires hairpin opening before ligation can ensue. In SCID cells, the generation of DSBs still occurs but the formation of coding joints is profoundly impaired, leading to an accumulation of coding *V(D)J* recombination intermediates. In contrast, SCID cells produce signal joints but with reduced efficiency (summarized in Bogue et al. 1998).

The inability of SCID mice to correctly process and ligate coding *V(D)J* intermediates suggested a link between *V(D)J* recombination and DSB repair. Indeed, SCID mice and cells derived from them are hypersensitive to IR, display defects in joining of IR-induced DNA DSBs, and fall into the IR sensitivity complementation group IR7 (Fulop and Phillips 1990; Biedermann et al. 1991; Hendrickson et al. 1991). Biochemical and genetic studies revealed a loss of DNA-PKcs function in SCID cells (Blunt et al. 1995; Kirchgessner et al. 1995; Peterson et al. 1995b). This results from a mutation in the DNA-PKcs gene that converts the Tyr-4046 codon into a stop codon, creating a truncated protein missing the last 83 amino acid of the kinase domain (Blunt et al. 1996; Danska et al. 1996; Araki et al. 1997). Although this mutated protein is highly unstable, it is still present at detectable levels (~5% of the levels of normal protein), raising the possibility that SCID cells retain residual DNA-PKcs function (Danska et al. 1996).

The pronounced effect of DNA-PKcs loss on coding but not signal joint formation in mice, and the accumulation of coding but not signal recombination intermediates in SCID cells suggests that there are mechanistic differences between coding and signal joint formation. Because coding but not signal ends go through a hairpin intermediate, DNA-PKcs might not be required for simple end-joining reactions but may be necessary for the processing of more complicated substrates before they can be ligated. For example, DNA-PKcs might help recruit or activate the Rad50/Mre11 complex, which is involved in NHEJ in yeast and can open DNA hairpins (Paull and Gellert 1998; Trujillo et al. 1998). Alternatively, DNA-PKcs might control the hairpin opening activities of the RAG1 /RAG2 complex (Zhu et al. 1996). Notably, hairpins bind to but do not activate DNA-PK (Smider et al. 1998). This raises the possibility that DNA-PK kinase functions are only triggered after the hairpin opening stage. Although the above models for DNA-PKcs function may be correct, recent work has indicated that, although there appear to be some cell-type specific differences, DNA-PKcs-deficient cells are often impaired to some degree in signal joint formation (Bogue et al. 1998; Errami et al. 1998b). Furthermore, the Arabian horse SCID mutant (equine SCID), which arose through loss of the carboxy terminal 967 amino acids of the DNA-PKcs protein (Wiler et al. 1995; Shin et al. 1997) is defective in both *V(D)J* signal and coding joint formation. On balance, the available data support the idea that the differential impact of DNA-PKcs loss on coding and signal joint formation is largely quantitative rather than qualitative.

Recently, knockout mice have been generated that are null for DNA-PKcs (DNA-PKcs$^{-/-}$; Jhappan et al. 1997; Gao et al. 1998) or ablated for its kinase domain (DNA-PKcs$^{kin-/-}$; Taccioli et al. 1998). Like SCID, these mice are immunodeficient and are severely defective in *V(D)J* coding but not signal joint formation. However, some phenotypic differences do appear to exist between these knockout mice and SCID animals. For example, DNA-PKcs$^{kin-/-}$ mice produce measurable CD4$^+$CD8$^+$ double-positive (DP) thymocytes, in contrast to barely detectable levels of these in the SCID mouse (Taccioli et al. 1998). The DNA-PKcs $^{-/-}$ mice generated by the chance integration of a yeast transgene into the DNA-PKcs gene, also produces DP thymocytes (Jhappan et al. 1997) but DP thymocytes were not reported in the other DNA-PKcs$^{-/-}$ mouse (Gao et al. 1998). Another difference is that the DNA-PKcs knockout mice of Jhappan et al. (1997) but not SCID mice show a significantly increased incidence of thymic lymphoma (see below). Interestingly, sublethal irradiation of SCID animals also leads to an elevation of DP thymocytes and thymic lymphoma (Danska et al. 1994). These data are consistent with the idea that the SCID mouse retains some residual DNA-PKcs function, and/or there is an alternative error-prone pathway(s) for resolving *V(D)J* recombination intermediates that can lead to the generation of oncogenic chromosomal translocations.

Mice have also been produced that are defective in Ku70 or Ku80 (Nussenzweig et al. 1996; Zhu et al. 1996; Gu et al. 1997; Ouyang et al. 1997). Consistent with biochemical studies, the loss of one Ku subunit leads to undetectable or severely reduced levels of the other and

to undetectable Ku DNA end-binding activity (Gu et al. 1997). Also as predicted, Ku70- and Ku80-deficient animals are very alike phenotypically and share many features with SCID and DNA–PKcs knockout mice. Thus, Ku-deficient animals are hypersensitive to IR (Nussenzweig et al. 1997). Furthermore, the dose of IR required for 50% mortality of Ku80$^{-/-}$ adult mice (2–4 months old) is similar to that for SCID mice (Biedermann et al. 1991). Younger Ku80$^{-/-}$ animals, however, are reported to be significantly more hypersensitive to IR than SCID mice (Nussenzweig et al. 1997). Ku-deficient animals also have severe combined immunodeficiency, and fail to generate functional T and B lymphocytes effectively, at least in large part due to defects in the ligation-dependent stages of *V(D)J* recombination (Nussenzweig et al. 1996; Zhu et al. 1996). In contrast to SCID and DNA–PKcs-null mice, however, signal joint formation is also highly defective in Ku-negative animals. These findings are in line with data from Ku80-deficient rodent cell lines (e.g., *xrs6*), which have revealed severe defects in both signal and coding joint formation (Pergola et al. 1993; Taccioli et al. 1993, 1994). A detailed analysis of one Ku80$^{-/-}$ mouse revealed that both blunt full-length signal ends and hairpin coding ends accumulate (Zhu et al. 1996; Han et al. 1997), suggesting that Ku is not required to protect the ends of these intermediates from degradation and, instead, may help recruit the appropriate ligation machinery and/or help to dissociate the RAG–DNA complex before end ligation can ensue.

A characteristic frequently observed in mice deficient in DNA–PK components is lymphoma. Early studies revealed that SCID mice have a low but significantly elevated incidence of lymphoma (Custer et al. 1985). Moreover, irradiation of SCID mice with sublethal doses of IR results in some rescue of T-cell development and thymic lymphoma in all animals (Danska et al. 1994). Similarly, SCID animals bred to possess homozygous mutations in p53 also display rescued T-cell development and an increased incidence of lymphoma/leukemia (Bogue et al. 1996; Guidos et al. 1996; Nacht et al. 1996). Taken together, these data suggest the existence of alternative recombination pathways, which are normally repressed, that are able to resolve *V(D)J* recombination intermediates and can lead to T-cell tumorigenesis. Consistent with this idea, loss of PARP, a known anti-recombinogenic factor, also results in rescue of T-cell development and T-cell lymphoma in SCID backgrounds (Morrison et al. 1997). Recently, complete penetrance of T-cell lymphoma has been demonstrated for animals knocked out for DNA–PKcs function (Jhappan et al. 1997), and a very similar phenotype exists for Ku70$^{-/-}$ mice, where the majority of animals develop T-cell lymphoma (Gu et al. 1997; Li et al. 1998). Fibroblasts derived from Ku70$^{-/-}$ mice form foci in culture and harbor chromosomal aberrations indicated by elevated levels of sister chromatid exchanges (Li et al. 1998). Furthermore, Ku70$^{-/-}$ cells derived from transformed foci are able to produce tumours in nude mice. To date, however, there is no report of lymphoma in Ku80$^{-/-}$ mice.

How is it that loss of DNA–PKcs or Ku70 can lead to lymphoma in mice whereas loss of Ku80 apparently does not? One hint may come from the observation that residual coding joints are detected and some mature T lymphocytes develop in Ku70$^{-/-}$ animals and in older SCID mice, but these appear to be fully absent in Ku80$^{-/-}$ mice. Although other effects on T-cell maturation or tumorigenesis cannot be discounted, an attractive model is that, in the absence of Ku70 or DNA–PKcs, normal constraints on the reactivity of DNA DSBs generated during *V(D)J* recombination are released. This could allow the DNA ends to undergo recombination reactions that are independent of the DNA–PK system; this would lead to some rescue of *V(D)J* recombination but might also result in inappropriate recombination events with other loci, some of which may have oncogenic capacity. If this is correct, one would have to postulate that the alternative recombination pathways are suppressed by DNA–PKcs or Ku70 but not by Ku80, and/or that these alternative pathways employ Ku80. In light of this hypothesis, it will be of interest to see whether lymphomas arise in DNA–PKcs$^{-/-}$, Ku80$^{-/-}$, or in Ku70$^{-/-}$, Ku80$^{-/-}$ double mutant mice, and whether the lymphomas generated in DNA–PKcs mutant or Ku70$^{-/-}$ backgrounds contain oncogenic chromosomal translocations involving T-cell receptor loci. The fact that Ku70 and DNA–PKcs act as T-cell tumor suppressors in mice raises the possibility that this may also be so in humans. Hinting that this may indeed be the case, citing unpublished data, Li et al. (1998) have reported that 6 out of 17 human lymphomas they tested were found to harbor mutations in the core domain of Ku70 that is implicated in heterodimerisation with Ku80 and DNA end binding. However, no mutations in DNA–PKcs have been found in human cancers to date.

In line with the existence of multiple DNA DSB repair pathways is the finding that loss of DNA–PKcs function can have different effects in different cell types. In contrast to cells from most tissues in the SCID mouse and fibroblast cell lines derived from SCID mice, DNA–PKcs$^{kin-/-}$ or DNA–PK$^{-/-}$ mice, embryonic stem (ES) cells established from the DNA–PKcs$^{-/-}$ mouse are not significantly radiosensitive (Gao et al. 1998). This implies that, unlike the situation in other cell types analyzed, the principal DNA DSB repair pathway(s) operating in ES cells is not dependent on DNA–PKcs. Part of the explanation for this may be that ES cells and possibly certain other stem-cell types employ homologous recombination instead of NHEJ to repair IR-induced DSBs. This is unlikely to be the whole explanation, however, because Ku70$^{-/-}$ ES cells are reported to be radiosensitive (Gu et al. 1997a), implying that ES cells use a repair pathway that is Ku70-dependent but DNA–PKcs-independent. Other evidence for such a pathway is the report that Ku-deficient but not DNA–PKcs-deficient cell lines are hypersensitive to etoposide, a topoisomerase II inhibitor that generates DNA DSBs (Jin et al. 1998).

Visually, the most striking phenotype of the Ku-deficient mice is their size: they are only 40%–60% of the weight of control littermates (Nussenzweig et al. 1996;

Gu et al. 1997b; Ouyang et al. 1997). The observed proportional dwarfism is not due to a reduction in cell size but to a reduction in cell number. Early passage embryonic fibroblasts derived from Ku-deficient animals grow approximately twice as slowly as controls, at least in part due to an increase in the proportion of nondividing but still metabolically active cells in the Ku-deficient cell populations (Nussenzweig et al. 1996; Gu et al. 1997b). A further examination of the impaired cell growth phenotype of Ku80$^{-/-}$primary fibroblasts revealed that they sustain DNA damage, arrest at the G_1/S or G_2/M checkpoints, and then fail to re-enter the cell cycle (Nussenzweig et al. 1996). One explanation for these findings is that Ku-deficient cells have functional DNA damage-induced cell cycle checkpoint mechanisms but fail to re-enter the cell cycle as a result of their inability to repair the damaged DNA effectively. The observed early appearance of nondividing cells in the absence of Ku has been taken to indicate a potential correlation between senescence and defective DNA DSB repair. However, the dwarf phenotype and apparent replicative senescence have not so far been reported for any of the DNA–PKcs mutant animals, raising the possibility that these phenomena relate to other, as yet undefined, functions of the Ku heterodimer, such as controlling cell growth or affecting telomeric structure (see above). The generation and analysis of mice deficient in DNA–PKcs, Ku70 or Ku80 has reinforced the conclusion that the enzyme complex they comprise plays a key role in the detection and repair of DNA DSBs. However, the apparent significant differences in phenotypes between the different animals, most notably, the differences in cancer incidence and animal size, raise the intriguing prospect that the different components of the DNA–PK enzyme have independent additional roles. Such roles could include influences on transcription, chromatin, or, as discussed below, telomeric structure. Nevertheless, it remains a possibility that some of the difference between the various mutant mice reflect different mouse genetic backgrounds and/or differences in housing conditions between the various laboratories.

Animal models have also been valuable in identifying additional functions for DNA–PKcs and Ku in the process of immunoglobulin heavy chain class switching, the process by which B lymphocytes change the constant region of their antibodies without altering their antigen-binding specificity. Class switching is a programmed genomic rearrangement in which site-specific recombination proceeds between repetitive DNA segments that flank the 5′ sides of the heavy chain constant regions, resulting in exchange of the IgM constant region for that of another antibody class (Harriman et al. 1993). Reminiscent of *V(D)J* recombination, this process can result in deletion of intervening DNA segments, and appears to be linked to the generation of site-specific DNA DSBs (Wuerffel et al. 1997). Despite the initial suggestion that switch recombination may proceed by homologous recombination, it is not impaired by loss of the homologous recombination component Rad54 (Essers et al. 1997). Instead, recent work has indicated that switch recombination is highly dependent on DNA–PKcs (Rolink et al. 1996), Ku70 (Manis et al. 1998) and Ku80 (Casellas et al. 1998). Although the mechanistic basis for switch recombination is still unclear, it seems likely that, like *V(D)J* recombination, it will proceed by a cut-and-paste pathway involving components of the NHEJ apparatus. The results from the class switching studies raise the exciting prospect that the DNA–PKcs–Ku system is also involved in other genomic rearrangements and related events in eukaryotes. In line with this proposal, work in *Drosophila* has revealed a function for Ku in P-element transposition (Beall and Rio 1996).

Role of Ku in telomere maintenance

Telomeres are specialized structures situated at the natural ends of linear eukaryotic chromosomes, and serve as caps to prevent chromosomal termini from being degraded by endogenous nucleases or being recognized as DNA damage (for review, see Shore 1998). In most eukaryotes, telomeres consist of tandem copies of simple repeat sequences that are synthesized through a non-DNA-templated mechanism by the enzyme telomerase. Recently, the use of *S. cerevisiae* as a system to study Ku function has revealed a role for this protein in telomere length maintenance. Initial work revealed that yeast defective in either Ku subunit have shortened telomeres; this corresponding to a loss of ~60% of the telomeric repeats (Boulton and Jackson 1996; Porter et al. 1996). At first, this was seen as somewhat puzzling—why would the yeast cell want to recruit a DNA NHEJ repair factor to telomeres, because telomeres actually protect chromosomal termini from engaging in NHEJ events? It now appears that the function of Ku in telomere length control is, at least in part, independent of its role in the NHEJ pathway, as disruption of the gene for ligase IV, which causes a DSB repair defect essentially the same as that in the absence of Ku, does not lead to telomere shortening (Teo and Jackson 1997). It has also been shown that Ku does not function in the same telomere maintenance pathway as telomerase nor as the Rad50p/Mre11p complex (Nugent et al. 1998).

Although the precise function of Ku in telomeric functions is as yet unclear, Ku does appear to be associated physically with telomeric DNA, and regulates the precise structure of telomeric termini, possibly via controlling the access of nucleases and recombinases (Gravel et al. 1998; Polotnianka et al. 1998). In addition, recent studies have shown that Ku is involved in the tethering of yeast telomeres to the nuclear periphery (Laroche et al. 1998). Yeast defective in Ku have also been found to be defective in telomeric silencing, the process in which genes placed in the vicinity of a telomere are subject to transcriptional repression (Boulton and Jackson 1998; Laroche et al. 1998; Nugent et al. 1998). An attractive explanation for the available data on telomeric silencing is that Ku helps to establish the transcriptionally silent heterochromatin-like state that normally exists in yeast telomeric DNA. Consistent with this proposal, Yku70p has been shown to interact in the yeast two-hybrid assay

with Sir4p (Tsukamoto et al. 1997). Sir4p forms a complex with Sir2p and Sir3p and, through associations with Rap1p and the amino termini of histones H3 and H4, can establish a heterochromatin like structure at the telomere (for review, see Grunstein 1997). It will clearly be of great interest to establish whether Ku functions similarly in higher eukaryotes.

Future directions

Through a combination of biochemical, cell biological, and genetic approaches, it has become clear that DNA–PK is a key player in DNA DSB repair. In addition, the available evidence suggests that DNA–PK components are involved in other processes, such as controlling chromatin structure and telomeric integrity. With the rich resources of reagents that are now available and the intense interest in this fascinating enzyme, one can confidently predict that there will be rapid progress towards gaining an even better understanding of DNA–PK and its associated proteins in the next few years. Of particular importance will be the identification of novel DSB repair components that operate in conjunction with DNA–PK, and studies investigating the links between DNA–PK and processes such as telomere structure, chromatin and transcription. Also of great importance will be the detailed determination of the structure of DNA–PK and its associated components, and establishing precisely how the DNA–PK enzyme is activated by DNA damage. In addition to providing insights into DNA–PK-associated functions, such studies are likely to provide important information into how structurally and functionally related kinases, such as ATM and ATR, function in the recognition and signaling of genomic damage.

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